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Sensitive and Specific Detection of Staphylococcal Epidermolysins A and B in Broth Cultures by Flow Cytometry-Assisted Multiplex Immunoassay

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Two of the most common bacterial skin infections of young infants and children are bullous impetigo due to *Staphylococcus aureus* and its more acute form, staphylococcal scalded skin syndrome. Epidermolysin A (ETA), ETB and, possibly, ETD are responsible for these diseases, which may appear as epidemics in pediatric patients. We tested the reliability of a flow cytometry-assisted multiplex immunoassay (Bio-Plex system) for the detection of ETA and ETB. The Bio-Plex system was found to be highly specific and highly sensitive for toxin concentrations of between 2 and 80,000 pg/ml. The results of this assay were 100% identical to the results of a PCR-based method. We demonstrated that this test did not generate any cross-reactions with ETD-producing isolates. The level of detection of ETB by this test differed according to culture conditions and from isolate to isolate; these results must be taken into account for diagnostic purposes.

Impetigo accounts for 10% of bacterial skin infections in neonates and young children. About 30% of children with impetigo develop bullous impetigo due to *Staphyloccocus aureus* (15). Children 7 years old and younger, adults with renal failure, and immunosuppressed adults may develop a generalized form of bullous impetigo called staphylococcal scalded skin syndrome. These two diseases are caused by strains of *S. aureus* that produce exfoliative toxins (ETs) or epidermolysins (epidermolysin A [ETA] and ETB). Three epidermolysins have been characterized: these include ETA (2), ETB (13), and ETD (30). These toxins are able to split the superficial epidermis by cleaving the desmosomes of the granular cell layer. This splitting exposes patients to secondary infections by opportunistic pathogens (15). ETs are serine proteases that specifically target and cleave Dsg1 (1). Dsg1 is a member of the desmosomal cadherin family, which includes desmogleins and desmocollins. These molecules are essential for the proper functioning of desmosomes, which maintain the integrity of epithelial tissues. ETs cleave Dsg1 in one of its calcium-binding domains. The three toxins hydrolyze the peptide bond after the glutamic acid at position 381, which forms part of a specific sequence located between extracellular domains 3 and 4.

Samples from infected patients are frequently sent to laboratories for analysis, and the detection of epidermolysins is essential to limit the risks of colonization or spreading in pediatric hospital departments.

A flow cytometry-assisted multiplex particle-based immunoassay (Bio-Plex system; Bio-Rad, Hercules, Calif.) has been designed and may be used to characterize bacterial compounds and toxins (9, 22). The Bio-Plex technology (8) consists of a particle counter and two laser beams (hardware) as well as software that allows the simultaneous discrimination of beads of different colors and the recording of phycoerythrin-generated fluorescence associated with the beads. One hundred different colors can be distinguished, making it theoretically possible to quantify 100 proteins or ligands simultaneously in a single sample in a single well of a microtiter plate. The aim of this study was to evaluate the reliability of the Bio-Plex system for the identification and quantification of ETA and ETB in culture supernatants.

MATERIALS AND METHODS

***S. aureus* isolates and culture conditions.** Eighty-five independent *S. aureus* isolates were considered in this study; only one sample from a given patient family and only one isolate from a nursery were included per 1-month period. All isolates came from young children who had bullous impetigo and who were seen at Strasbourg University Hospital or Cayenne Hospital (French Guiana) between 1992 and 2002. These isolates included 25 non-epidermolysin-producing isolates, 26 ETA producers, 9 ETB producers, and 25 ETA and ETB producers, as determined by a simple Ouchterlony test (11) with rabbit polyclonal affinity-purified antibodies. Five isolates originating from devices implanted at Strasbourg University Hospital were included in this study because they carried the ETD gene. Each bacterial isolate was grown in 24-well microtiter plates filled with 0.6 ml of 2× tryptone-yeast extract (TY) broth, Mueller-Hinton broth, Trypticase soy broth, or yeast extract-Casamino Acids-pyruvate broth (10) for 16 h at 37°C in a 10% CO_2 atmosphere with vigorous shaking before the epidermolysin contents of culture supernatants were analyzed.

Toxin purification. *S. aureus* IBS-SA417 (ETA producer) and TC 142 (ETB producer) were grown overnight at 37°C with gentle shaking (150 rpm) in 2-liter Erlenmeyer flasks filled with 0.2 liter of 2× TY broth in a 10% CO_2 atmosphere. Cultures (total, 3 liters) were filtered with a Pellicon cassette system (pore size, 0.45 μm; Millipore). Supernatants were concentrated to 0.2 liter before being dialyzed against H_2O (cutoff, 10 kDa). ETA was further purified as described previously (5). ETB fractions were applied twice to a DEAE-Trisacryl M column (IBF Biotechniques, Villeneuve la Garenne, France) equilibrated with 10 mM Tris-HCl (pH 8.5). Proteins were eluted with a continuous gradient of NaCl (0 to 0.5 M). ETB fractions were pooled and further purified by alkyl-Superose chromatography as described for ETA (5) with a 2 to 0 M $(NH_4)_2SO_4$ gradient in 50 mM KH_2PO_4 (pH 7.0).

Antibody preparation. ETA and ETB (60 μg/ml) were denatured in 60 mM formaldehyde at 37°C for 48 h. Next, they were dialyzed against 50 mM sodium phosphate–150 mM NaCl (pH 7.5) and mixed with 50% (vol/vol) incomplete Freund's adjuvant to a final concentration of 20 μg/ml. The backs of New

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Zealand rabbits (F_2 females of 3 kg or more) were shaved, and 1 ml of the mixture was injected intradermally at five points (200 μl per injection) along the spine. The rabbits were injected every 4 weeks for 16 weeks, and then 25 ml of cardiac blood was collected. Blood was allowed to coagulate for 15 h at 6°C, and then serum was recovered and filtered (pore size, 0.22 μm). Specific antibodies were affinity purified by using Hitrap HP columns (Amersham-Pharmacia Biosciences, Orsay, France) that were *N*-hydroxy-succinimidyl activated with purified ETA and ETB for immobilization according to the manufacturer's recommendations. Affinity columns were equilibrated with 30 mM $NaHCO_3$–0.15 M NaCl (pH 7.5). Filtered rabbit serum (5 ml) was applied to the columns, and specific antibodies were eluted with 0.1 M glycine–0.2 M NaCl (pH 2.5) into tubes containing a 1/10 volume of 1 M Tris-HCl (pH 9.0).

Covalent binding of anti-ETA and anti-ETB antibodies to beads. Polystyrene beads (5.5-μm diameter; Bio-Rad) were colored with a mixture of red and infrared dyes. These fluorophores each can be combined with the others at 10 different concentrations, potentially defining 100 regions of colored beads. Beads of color regions 24 and 43 were coupled to rabbit polyclonal affinity-purified anti-ETA and anti-ETB antibodies, respectively. The coupling reactions included 9 μg of purified antibodies, 1.25×10^6 beads in 100 μl, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, and *N*-hydroxy-sulfosuccinimide as recommended in the instructions for the Bio-Plex amine coupling kit. At least 30% of the initial amounts of modified beads were finally recovered. The amounts recovered depended on the reactions and the centrifugation efficacies.

Biotin-labeled anti-ETA and anti-ETB antibodies. Each purified antibody (2 mg) was labeled for 1 h at 25°C with a 1/15 molar excess of sulfosuccinimidyl-6-(biotinamido)-hexanoate-biotin (Uptima) in 100 mM HEPES–150 mM NaCl (pH 7.5) in a total volume of 300 μl. After the labeling step, excess biotin and NaCl were removed by using a PD10 column (Pharmacia), and biotin-labeled antibodies were eluted with 20 mM HEPES–150 mM NaCl–1 mM EDTA (pH 7.2).

Evaluation of labeled beads. The beads were mixed and incubated with antigens for 1 h at 20°C in the dark, with soluble and biotin-labeled antibodies for 40 min at 20°C in the dark, and then with phycoerythrin for 10 min at 20°C in the dark. Labeling of the beads with soluble antibodies was checked at 532 nm by using a FacSort flow cytometer (Becton Dickinson, Rungis, France). All steps were performed with vigorous shaking. Each step was separated by three washes with 200 μl of 10.1 mM Na_2HPO_4–1.8 mM KH_2PO_4–140 mM NaCl–2.7 mM KCl (pH 7.0)–0.05% (vol/vol) Triton X-100 (PBST).

Calibration of sensitivity and validation of color regions. Prior to experiments, the Bio-Plex signal output must be standardized and the instrument performance must be validated. To optimize assay reproducibility by standardizing the signal and optimizing the laser output for low-range sensitivity, daily calibration with the Bio-Plex calibration kit is necessary. The Bio-Plex validation kit is used once per month to verify optical alignment, reporter channel performance, classification accuracy of the beads, and fluid integrity according to previously published procedures (8). These kits were used as recommended by the manufacturer.

Assay. After the wells of a porous, flat-bottom microtiter plate were wet with PBST coupled to a vacuum manifold, 2,500 coupled beads were placed in each well and then washed three times by vacuum filtration with 200 μl of PBST. Coupled beads were incubated with samples containing antigens for 1 h at 20°C in the dark with agitation, washed twice with PBST, incubated with 16 μg of detection antibody in 50 μl of PBST for 45 min at 20°C in the dark with vigorous shaking, and further washed three times with PBST. The beads were incubated with 100 ng of streptavidin-phycoerythrin in a total volume of 50 μl per well for 10 min at 20°C in the dark with shaking and then were washed twice with PBST. The beads were resuspended and analyzed by using the Bio-Plex suspension array system. The fluorescence intensity (arbitrary fluorescence units [FU]) of 50 or 100 beads was recorded by using integrated Bio-Plex manager software. The analysis is quicker when only 50 events are recorded, but statistical calculations are significant in both situations.

DNA extraction. Isolates were grown in 1 ml of 2× TY broth at 37°C overnight and then centrifuged for 10 min at 5,000 × *g*. The pellet was suspended in 180 μl of an enzymatic solution (200 μg of lysostaphin/ml, 20 mM Tris-HCl, 2 mM EDTA [pH 8.0], 1.2% [vol/vol] Triton X-100) and then incubated for 30 min at 37°C. Proteinase K (Qiagen) and 200 μl of AL buffer (Qiagen) then were added. After 30 min at 56°C and 15 min at 95°C, the lysates were centrifuged. DNA was extracted from the bacterial pellet by using a QIAmp DNA minikit (Qiagen) according to the manufacturer's recommendations.

PCR amplification of *eta*, *etb*, *etd*, and *hlgC*. The following primers were used in this study: *hlgC* forward, 5′-ATTAGATTGTGAACTGATAGACACACAG TATTTTGCACCCCAATATATTTTT-3′, and reverse, 5′-ACACTTAGGATC CGCCAATGATACTGAAGACATCGGTAA-3′; *eta* forward, 479–5′-ATGGT GTCAATGCATTTAATTTACCA-3′-503, and reverse, 925–5′-CTTTATGATC GAATGGATAGCCTAT-3′-901; *etb* forward, 513–5′-GCAAAAGAATACAG CGCAGAAGAAATC-3′-539, and reverse, 840–5′-ACGGAGATTCTTTAATT TCTTCAGCT-3′-814; and *etd* forward, 5′-CACGAATTCAATACATATGAA GAATCTGAAATTTTA-3′, and reverse, 5′-TGCAGAATTCAAGTTATTCCA TAATGATTAGAATGA-3′. PCR was performed with 0.4 pM each primer, 3 mM $MgCl_2$, 5 μl of FastStart *Taq* DNA polymerase, 10× buffer (Roche Diagnostics), 0.15 nM deoxynucleoside triphosphate, 2 U of FastStart *Taq* DNA polymerase (Roche Diagnostics), and 1 μl of a 1/200 DNA dilution in a total reaction volume of 49.4 μl. Amplification was performed by using a Perkin-Elmer 9700 thermocycler with an initial denaturation step at 92°C for 1 min; 35 cycles at 92°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and a final step at 72°C for 3 min. Amplification products were separated on a 0.6% (wt/vol) agarose gel and stained with ethidium bromide before being analyzed on a UV bench by using GelDoc2000 (Bio-Rad).

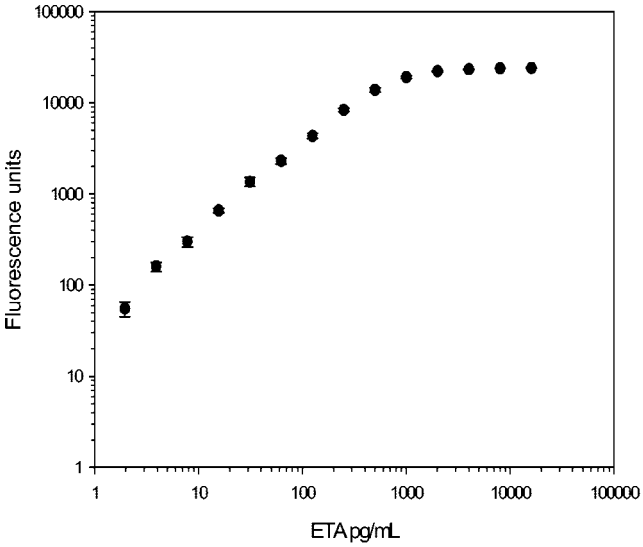

FIG. 1. Standard curve for the quantification of ETA with the Bio-Plex system. A 4.5-log-unit range of concentrations of ETA were studied. Error bars indicate standard deviations ($n = 4$). Nonlinear regression of this curve was used to estimate ETA concentrations in culture supernatants.

RESULTS

Optimization of quantities of beads and antibodies. We tested various quantities of beads (1,000 and 2,500 beads/200 μl) and various concentrations of biotinylated antibodies (1, 2, 4, 8, and 16 μg/200 μl). It took a long time to read samples when 1,000 beads/assay were used. The analysis was quicker with 2,500 beads/assay. The system was less sensitive with 2 μg of biotinylated antibodies (15,000 and 12,500 FU for ETA and ETB at 1 ng/ml, respectively) than with 8 or 16 μg of antibodies (21,000 and 18,000 FU for ETA and ETB at 1 ng/ml, respectively). For optimal sensitivity and security under all conditions, an antibody concentration of 16 μg/200 μl and 2,500 beads/assay were chosen.

Sensitivity. We tested dilutions of purified toxins (ETA and ETB) ranging from 0.5 pg/ml to 16 ng/ml. A fluorescence signal was detected at 532 nm for both proteins at concentrations exceeding 2 pg/ml (55 and 70 FU for ETA and ETB at 2 pg/ml, respectively) (Fig. 1 and 2). These values were considered significantly different from the background value by the calculating system. A saturating signal was reached at 22,000 FU. The calibration curves for ETA and ETB were linear over

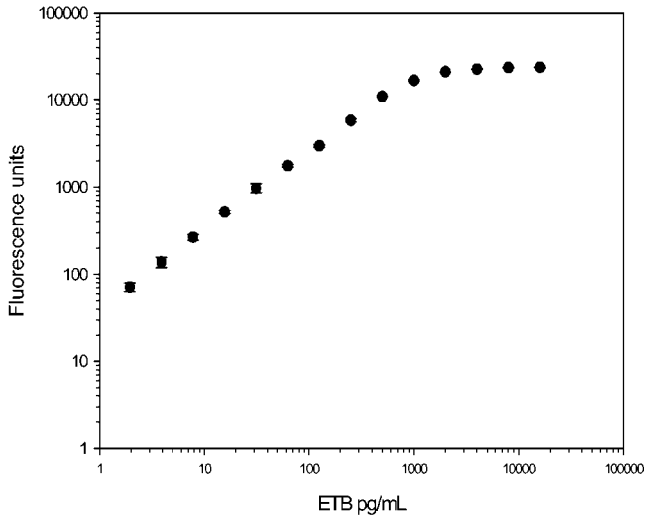

FIG. 2. Standard curve for the quantification of ETB with the Bio-Plex system. A 4.5-log-unit range of concentrations of ETB were studied. Error bars indicate standard deviations ($n = 4$). Nonlinear regression of this curve was used to estimate ETB concentrations in culture supernatants.

more than 2.5 logarithmic magnitudes at between 2 and 1,000 pg of toxin/ml (Fig. 1 and 2).

The amount of toxin in the culture supernatants saturated the system, making it necessary to dilute the supernatants. Analysis of these dilutions revealed that the ETA concentration ranged from 60 to 800 μg/ml and that the ETB concentration ranged from 18 to 88 μg/ml. When diluted 1/50,000 or 1/200,000, ETB-producing isolates never saturated the fluorescence signal. However, some ETA-producing isolates produced a saturating signal even at a 1/200,000 dilution. This result was due to a difference in the levels of expression of the genes encoding these two toxins. The use of different culture media did not significantly affect the fluorescence signal, and the same dilutions worked equally well in these culture media, phosphate-buffered saline, or normal rabbit serum. However, when the isolates were grown in a normal atmosphere (i.e., not CO_2 enriched), the levels of toxins were 200- to 1,000-fold lower than those observed under optimized conditions.

Specificity. We tested each possible combination for true- or false-positive results in sandwich assays in the presence of high antigen concentrations (up to 80,000 pg/ml) (Table 1). All heterologous combinations were negative, except for the combination that corresponded to anti-ETB antibodies coupled to beads (anti-ETB beads) plus 80 ng of ETA, as revealed by soluble anti-ETA biotinylated antibodies. This combination gave a fluorescence signal equivalent to that seen with 3 pg/ml of ETA with anti-ETA beads. Due to the saturation of signals with toxin-producing isolates and the lack of a signal with other isolates, each culture supernatant was diluted 1/50,000 and 1/200,000 before testing to overcome the risk of obtaining a false-positive result without affecting sensitivity.

TABLE 1. Specificities of anti-ETA or anti-ETB beads for ETA and ETB

Heterologous combination[a]	FU
Anti-ETA beads + ETB + anti-ETA antibodies	ND[b]
Anti-ETA beads + ETB + anti-ETB antibodies	ND
Anti-ETA beads + ETA + anti-ETB antibodies	ND
Anti-ETB beads + ETA + anti-ETB antibodies	ND
Anti-ETB beads + ETA + anti-ETA antibodies	130
Anti-ETB beads + ETB + anti-ETA antibodies	ND

[a] Range of heterologous antigen concentration 1 to 80,000 pg/ml.
[b] ND, not detectable.

TABLE 2. Correlation between results of the multiplex immunoassay (Bio-Plex system), the immunoprecipitation assay, and PCR tests for ETA and ETB

Test	No. of isolates classified as follows:			
	ETA^+ and ETB^+	ETA^- and ETB^+	ETA^- and ETB^-	ETA^- and ETB^-
Immunoprecipitation	26	9	25	25
Bio-Plex system	27	9	24	25
PCR (*eta* and *etb*)	27	9	24	25[a]

[a] Including one etd^+ isolate.

We tested all of the *S. aureus* isolates used in this study for the presence of the *eta*, *etb*, and *etd* genes, which encode ETA, ETB, and ETD, respectively. We also tested all isolates for *hlgC*, which encodes the ubiquitous HlgC component of the gamma-hemolysin, as a positive control (20). All 85 isolates were *hlgC* positive ($hlgC^+$), but only 24 appeared to be *eta* positive (eta^+) and *etb* negative (etb^-) and 27 appeared to be eta^+ and *etb* positive (etb^+). Immunoprecipitation revealed that 25 isolates were ETA^+ and ETB^- and 26 were ETA^+ and ETB^+ (Table 2). The additional eta^+ and etb^+ isolate was confirmed to produce ETB by the Bio-Plex system. The culture supernatant of this isolate contained 3.5 μg of ETB/ml, whereas the ETB concentration in the supernatants of the other ETB^+ isolates detected by immunoprecipation were between 18 and 89 μg/ml (mean, 46 μg/ml).

Only one isolate found to be ETA negative (ETA^-) and ETB^- by the immunoprecipitation, Bio-Plex, and amplification procedures was found to be *etd* positive (etd^+) (Fig. 3). We therefore tested five other independent isolates obtained from catheter infections, previously typed as etd^+, and typed as ETA^- and ETB^- by immunoprecipitation. These isolates remained PCR negative for the *eta* and *etb* genes. These five isolates were also found to be ETA^- and ETB^- by both immunoprecipitation and the Bio-Plex system, indicating that the ETA and ETB assay did not generate any cross-reactions with ETD-producing isolates.

Reproducibility. Results were highly reproducible from well to well regardless of whether the wells were treated simultaneously or not. They were also highly reproducible on different microplates and when three different concentrations of epidermolysins (10, 50, and 200 pg/ml) were tested over 4 weeks with the same batch of labeled beads. Furthermore, when a standard titration curve was used, a given dilution of antigens provided results with a correlation factor (r^2) of 99.5%. Variations were observed only when we compared three batches of beads tagged with antibodies at different times (Table 3). Under these conditions, concentrations varied by up to 15% when toxins diluted to concentrations of between 1 and 60 pg/ml

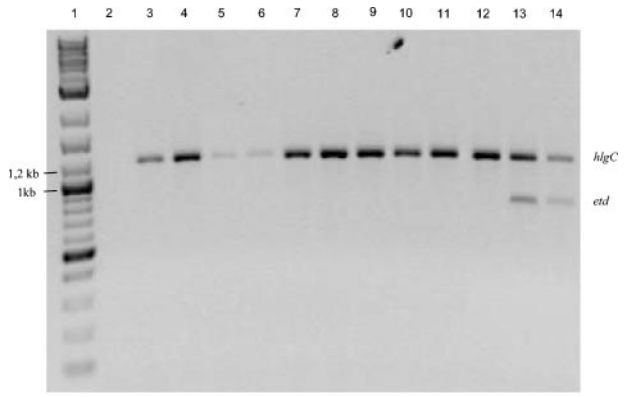

FIG. 3. PCR amplification products for total DNAs from 12 representative *S. aureus* isolates originating from bullous impetigo cases in this study. *etd* was coamplified with the *hlgC* control. Lane 1, DNA ladder; lane 2, negative control (water); lanes 3 to 13, *S. aureus* isolates; lane 14, *etd*$^+$ *S. aureus* isolate.

were used. The results were unaltered when the toxins were diluted in 9‰ (wt/vol) NaCl, 2× TY medium, or Mueller-Hinton medium or when isolates were grown in 2× TY, Mueller-Hinton, or Trypticase soy medium.

DISCUSSION

The detection of staphylococcal epidermolysins is important because of the risk of secondary infections and/or sepsis and because of the risk of infection spreading in pediatric wards. The "gold standard" identification method initially was the intradermal injection of culture supernatants into newborn mice (12, 19). Electrically assisted or nonassisted Ouchterlony tests can also be used to assess the distributions of ETA- and ETB-producing isolates (6, 14, 23, 24). However, these methods are limited in sensitivity and can give false-negative results for isolates that produce small amounts of toxins or that express toxins differently (26). It has long been known that the culture atmosphere must be enriched with 10% CO_2 to obtain large amounts of secreted epidermolysins (3, 24, 26). This CO_2-dependent secretion suggests that the expression of the epidermolysin genes is dependent on the pH or oxidizing environment of cultures (29, 31).

Several different PCR tests that can detect the epidermolysin genes but not necessarily their levels of expression have been developed (12, 16, 21). A number of phenotypic tests have also been developed to enhance sensitivity and specificity. Western blotting, $F(ab')_2$ enzyme-linked immunosorbent assays (ELISAs), double-antibody ELISAs, and immunodiffusion assays have been compared (16). Western blotting was found to provide results most similar to those of PCR, although it is more time-consuming than the other assays. Immunodiffusion assays generally lack sensitivity and require large amounts of antigen (≥5 μg/ml); the amount of antigen required probably depends in part on the quality and purity of recovered antibodies. However, ELISAs with complete antibodies generated some false-positive results, possibly because of the presence of protein A in *S. aureus* culture supernatants (16). Protein A can be found in culture supernatants of disregulated isolates or because of abundant bacterial autolysis. Protein A has been reported to bind to the Fc domain of antibodies but not to several Fc fragments of immunoglobulin G (27, 28). Therefore, phenotypic tests based on the occurrence of a molecular network overcome the potential effect of protein A.

TABLE 3. Reproducibilities of tests for ETA and ETB with three batches of beads

Range of ETA or ETB concn (pg/ml)	Coefficient of variation (± %)[a]
2,000–16,000	<2
60–2,000	<8
1–60	8–15

[a] For 10 samples.

The affinity-purified antibodies used in the Bio-Plex system in this study appeared to be able to distinguish between ETA- and ETB-producing isolates, providing results identical to those of PCR. However, according to the Ouchterlony test, one isolate produced ETA only, whereas both the *etb* probe and the Bio-Plex system suggested that this isolate produced ETB. These results illustrate variations in the production of ETB, although the same results were not obtained for 51 ETA-producing isolates.

The Bio-Plex system is highly sensitive, detecting approximately 1 pg of epidermolysin per ml (4×10^{-14} M), suggesting that it could be used for the direct detection of antigens present in clinical specimens. ETA and ETB have similar three-dimensional structures and share about 42% sequence identity; however, they can be specifically detected by polyclonal antibodies. Anatoxin-generated antibodies do not induce significant cross-reactions at this level of sensitivity. Specificity is satisfactory, and only large amounts of ETB may produce a signal with anti-ETA antibodies; this situation can be avoided by diluting culture supernatants. We showed that this flow cytometry-assisted immunoassay can be used to detect two related toxins in a single assay. It certainly has greater potential. This type of assay directed toward epidermolysins could be completed with anti-ETD antibodies. When various staphylococcal toxins and virulence factors are considered, the multiplex aspect of this system remains interesting for clinical surveys and investigations and for studying physiopathology directly. In practice, such an assay necessitates antibodies directed against at least two different epitopes of an antigen provided that these antibodies are specific enough. Theoretically, immunoglobulins appear to be unsuitable, but $F(ab')_2$ may be useful. Antibodies may also be directed against single epitopes, linear peptide sequences, or complete proteins.

Each well of a flat-bottom microtiter plate represents one possible multiplex immunoassay. We obtained similar results with the same batches of labeled beads and biotinylated antibodies for at least 1 month. Internal calibration with control dilutions of antigens is needed when different batches are used, as for other quantifying tests. The method provided results within 5 h of isolate identification and growth under specific conditions; this time is not too long when multiple toxin identification and antibiograms are requested. Fusidic acid- and methicillin-resistant isolates were recently found to cause impetigo (18). Thus, it is important to distinguish between streptococci and staphylococci for diagnosis and further to deter-

mine the antibiograms of isolates to allow optimal treatment. To date, PCR-assisted detection of epidermolysin-encoding genes has been reported only after isolation of staphylococci (3, 4, 14, 17). Injected epidermolysins have been shown to persist in serum in mice (25), as have epidermolysin-producing isolates from patients with atopic dermatitis (29). The ability to detect these toxins directly in bulla fluids and possibly in sera might be of interest.

In conclusion, flow cytometry-assisted multiplex immunoassays are promising and sensitive methods for the simultaneous and rapid detection of various antigens or antibodies contained in a single specimen (7). This technology should be tested with clinical samples. Its versatility makes it an attractive alternative to other tests.

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